Screening antimicrobial activity of tropical edible medicinal plant extracts against five standard microorganisms for natural food preservative

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Abstract

Edible medicinal plants are often used in the treatment of various ailments and spice in traditional food preparation. In this study, 45 of tropical edible medicinal plants extracts from Indonesia, Malaysia, and Thailand were screened for their antimicrobial activity against five standard microorganisms for food preservative namely Aspergillus niger, Candida albicans, Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus. The methanol extracts of Piper nigrum L. seed, Piper cubeba L. seed, and the root of Ligusticum acutilobum Siebold and Zucc. showed antimicrobial activity against five species of standard microorganisms. Among them, P. cubeba L. extract demonstrated the most susceptible against all tested microorganisms. Minimal inhibitory concentration (MIC) and minimal bactericidal or fungicidal concentration (MBC or MFC) were performed by the broth microdilution techniques as described by the Clinical and Laboratory Standard Institute. MIC values of P. cubeba L. extract to A. niger, C. albicans, E. coli, P. aeruginosa and S. aureus were 12.8, 1.6, 3.2, 6.4, and 1.6 mg/ml, respectively. P. cubeba extract killed A. niger, C. albicans, E. coli, P. aeruginosa and S. aureus with MBC values of 25.6, 3.2, 6.4, 12.8, and 3.2 mg/ml, respectively. The potent antimicrobial activity of P. cubeba L. extract may support its use for natural food preservative.

Keywords

Antimicrobial
Food preservative
Medicinal plants
Piper cubeba

Introduction

The growth of bacteria, yeast, and mould in foods and food products results in waste products and is costly as well as sometimes hazardous. Many different bacterial and fungal species can spoil food products or produce toxins or both. Several food preservation systems such as heating, refrigeration and addition of antifungal compounds can be used to reduce the risk of outbreaks of food poisoning; however, these techniques frequently have associated adverse changes in organoleptic characterizations and loss of nutrient (Valero and Frances, 2006). Although chemical preservatives prevent microbial growth, their safety is questioned by a growing segment of consumers. Moreover, consumer demand of natural, fresh, chemical-additive free and safe food products is increasing at the present (Gould, 1996). Recently, there is interest in the development of natural preservative from edible medicinal plant extracts (EMPE) (Singh et al., 2010). Thus, the properties of tropical EMPE for natural food preservative need to be investigated in order to prevent microbial spoilage and therefore to prolong the shelf life of the food or food products, and finally to protect the consumers from potential infection.

Edible medicinal plants are used widely in the food industry as flavors and fragrances, also exhibit useful antimicrobial properties (Rios and Recio, 2005). Many plant-derivate antimicrobial compounds have a wide spectrum of activity against foodborne pathogens and this has led to suggestions that they could be used as natural preservatives in foods (Smith-Palmer et al., 1998; Cho et al., 2008). The safest way to look for natural food preservative is to search for activity against classes of standard microorganisms. These include Escherichia coli and Pseudomonas aeruginosa (Gram positive), Staphylococcus aureus and Bacillus cereus (Gram negative), Candida albicans (yeast), Aspergillus flavus and A. niger (moulds) (Dweek, 1997).

The objective of this study is to screen the antimicrobial activity of tropical EMPE from Indonesia, Malaysia, and Thailand against standard five species microorganisms mentioned above. The susceptibility of selected tropical EMPE in term of minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC), on the five standard microorganisms will be determined using CLSI methods (Clinical

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Materials and Methods

Plant materials
The tropical edible medicinal plants were collected from traditional market of Indonesia (IN), Malaysia (MY) and Thailand (TH) and identified by Biopharmaca Research Center (BRC), Bogor Agriculture University (IPB) (Bogor, Indonesia), Institute of Bioscience, Universiti Putra Malaysia (Selangor, Malaysia), and Institute of Science, Walailak University (Nakhon Si Thammarat, Thailand), respectively. The voucher specimens are deposited in the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia (Table 1).

Plants extract preparation
The dried plants (100 g) were ground and extracted twice with 400 mL of 100% (v/v) methanol for 48 h at room temperature. Tropical edible medicinal plant extracts (EMPE) were filtered with Whatman filter paper NO.2 (Whatman International Ltd., Middlesex, England) and concentrated with a rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 50°C, yielding methanol crude extracts. Each methanol tropical EMPE was dissolved in 100% DMSO to obtain 1,024 mg/mL and the solution was dissolved in 1:10 (v/v) sterile double distilled water (ddH₂O) to obtain 102.4 mg/mL stock solutions. Final concentration of DMSO was 10% which was found not to kill the five standard microorganisms tested in this study.

Tested microorganisms and inoculum preparation
Aspergillus niger ATCC 2029, Candida albicans ATCC 10231, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 15692 were obtained from the American Type Culture Collection (Rockville, MD, USA). Staphylococcus aureus KCCM 11764 was obtained from Korean Culture Center of Microorganisms (Seoul, South Korea).

Aspergillus niger
A. niger was grown on PDA (Difco, Spark, MD, USA) at 35°C for 7 days. A standardized inoculum suspension of A. niger was prepared by the method of CLSI M38-A (CLSI, 2002). Briefly, A. niger was grown on PDA at 35°C for 7 days (Rukayadi and Hwang, 2007). Seven-day-old colonies were covered with approximately 1 mL of sterile 0.85% saline, and the suspensions were made by gently probing the colonies with the tip of a Pasteur-pipette. The resulting mixture of conidia or sporangiospore and hyphal fragments was withdrawn and transferred to a sterile tube. After heavy particles were allowed to settle for 3 to 5 min, the upper homogenous suspensions were collected and mixed with a vortex mixer for 15 s. The densities of the conidial suspensions were read and adjusted to an optical density (OD) that ranged 80 to 82% transmittance. These suspensions were diluted 1:50 in sterile water distilled water. The 1:50 inoculum dilutions corresponded to 2× density (approximately 0.4 × 10⁶ to 5 × 10⁶ cfu/mL) (Rukayadi and Hwang, 2007). Inoculum quantification was made by plating 0.01 mL of 1:100 dilution of the adjusted inoculum on Sabouraud dextrose agar (SDA) (Difco) to determine the viable number of cfu/mL. The plates were incubated at 28-30°C and observed daily for the presence of fungal colonies. The 2× conidial or sporangiospore inoculum suspension was approximately 5 × 10⁴ cfu/mL.

Candida albicans
The C. albicans was cultured in Sabouraud dextrose broth (SDB) or on Sabouraud dextrose agar (SDA) (Difco, Spark, MD, USA) for 48 h at 35°C. Meanwhile, inoculums suspension of C. albicans was prepared as follows: the C. albicans was propagated in SDB at 35°C for 24 h with 200 rpm agitation. One mL of 24 h old culture in SDB was centrifuged (3900 × g at 4°C for 1 min), and the pellets were washed twice with 1 mL of physiological saline. Sterile physiological saline was added to give a McFarland turbidity 0.5 at 530 nm, corresponding to 5 × 10⁶ cfu/mL (CLSI, 2002).

Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus
E. coli, P. aeruginosa and S. aureus in Mueller Hinton broth (MHB) or Mueller Hinton agar (MHA) (Difco, Franklin Lakes, NJ, USA). An inoculum cell suspension was prepared as follows: bacterial species was first grown aerobically on MHA plate for 24 h at 37°C. Subsequently, a single colony of each bacterial species was propagated in 10 mL of MHB at 37°C overnight with 200 rpm agitation. A quantity of 1 mL of overnight cultures in MHB was centrifuged (3,000 × g at 4°C for 1 min) and pellets were resuspended in 1 mL of MHB. Standardized inoculums (a McFarland standard) for each strain were 5 × 10⁶ cfu/mL. A standard curve of turbidity against colony forming unit (cfu) was used to obtain the number of cells.

Screening bioassay
Methanol extracts of 45 tropical medicinal plants were screened for antimicrobial activity using the standard paper disk diffusion assay (CLSI, 2002). 100 µL of inoculum of each standard microorganisms...
prepared as above was spread on SDA plates with a sterile cotton swab. Sterile filter paper discs, 6 mm diameter (Schleicher and Schuell, Dassel, Germany), were placed on the disks and 50 μL of 102.4 mg/mL (w/v) methanol extract of samples were loaded on the paper discs. 1 mg/mL of amphotericin B (AMB), a positive control for C. albicans and A. niger or chlorhexidine (CHX, a positive control for E. coli, P. aeruginosa and S. aureus), and 10% of DMSO (a negative control) were included in the assay. The plates were incubated at 37°C for 12-24 h for bacterial species and 24-48 h for fungi and observed for any clear zones. The experiments were preformed twice to verify the results.

**MIC and MBC or MFC determination**

*In vitro* susceptibility tests were performed in a 96-well microtiter plate to determine MIC and MBC or MFC of tropical EMPE against A. niger, C. albicans, E. coli, P. aeruginosa and S. aureus using standard broth microdilution methods with an inoculum of 5 × 10^4 cfu/mL for A. niger, and 5 × 10^6 cfu/mL for C. albicans and bacterial species, according to the guidelines of CLSI M7-A6 (for bacterial species) (CLSI, 2003), M27-A2 (for C. albicans) (CLSI, 2002) and M38-A (for A. niger) (CLSI, 2002). Briefly, a 2-fold EMPE stock solution or other antimicrobial agent preparations was mixed with the test organisms MHB, SDB, and PDB for bacterial species, C. albicans and A. niger, respectively. Column 12 of the microtiter plate contained the highest concentrations of EMPE or other antimicrobial agents, and column three contained the lowest concentrations of EMPE or other antimicrobials agents. Column 2 served as the positive control for all samples (only medium and inoculum or antimicrobial agent-free wells), and column 1 was the negative control (only medium, no inoculum, no antimicrobial agent). Microriter plates were incubated aerobically at 37°C for 24 h for bacterial species and 48 h for C. albicans and A. niger. The Mix was defined as the lowest concentration of antimicrobial agent that resulted in the complete inhibition of visible growth.

MBC and MFC values were determined for each of EMPE/microorganism species/medium combination as outlined for MIC by removing the media from each well showing no visible growth and subculturing onto MHA, SDA or PDA plates (Rukayadi et al., 2006; 2009; 2010). The plates were incubated at 37°C until growth was seen in the growth control plates. MBC or MFC were defined as the corresponding concentrations required to kill 100% of the microorganisms.

**Results and Discussion**

The susceptibility of tropical EMPE towards 5 standard species was tabulated on Table 1, based on their inhibition diameter on plates. Previous authors have described that an inhibition one of 14 mm or greater which include with diameter of disc was conceived as high antimicrobial activity (Parekh and Chanda, 2007). Based on the results, it showed that the tropical EMPE were more active against S. aureus. S. aureus is Gram positive bacterium while others, E. coli and P. aeruginosa are Gram negative bacteria. On the other hand, A. niger and C. albicans are both fungi. The results shows in agreement with previous study which indicated that plant extracts were more active against Gram positive bacteria than those of Gram negative bacteria (Kelmanison et al., 2000; Parekh and Chanda, 2007) (Table 1). Different species of plants influence its activity against microbes tested due to the different microbe cell wall compound (Grosvenor et al., 1995). Three plants extracts namely L. acutilobum, P. cubeba and P. nigrum showed the best potential antibacterial activity against of all microbes tested. Out of 45 tropical EMPE, C. xanthorrhiza extract has the strongest potential antimicrobial activity against A. niger and C. albicans. Moreover, O. basilicum, L. acutilobum and P. cubeba have the strongest antimicrobial activity against E. coli, P. aeruginosa and S. aureus, respectively. In this study, P. aeruginosa is the most resistant strain against all tropical EMPE tested. In contracts, S. aureus is the most susceptible strain among all microbe tested against all tropical EMPE (Table 1).

Table 2 shows the MIC and MBC or MFC values of L. acutilobum, P. nigrum and P. cubeba on A. niger, C. albicans, E. coli, P. aeruginosa and S. aureus. MICs and MBCs or MFCs of P. cubeba extract against five standard microbes exhibit relatively stronger than those of L. acutilobum and P. nigrum. The essential oil of P. cubeba contain hydrocarbon terpene and oxygenated terpene, thus, could be used as antioxidant (Hwang et al., 2005), antibacterial (Feng et al., 2009) and antifungal (Yang et al., 2010). P. nigrum is used to treat various diseases and has shown to have antimicrobial activity (Rahman et al., 2011).

The major phytochemical present in the crude extract of P. nigrum was found to be piperine, the active constituent showing inhibitor effect in the crude extract. The fresh berry oil of P. nigrum L. recorded MIC values were 2.5 mg/mL against P. aeruginosa and 8.5 mg/mL for A. niger whereas the dry berry oil needed 4 mg/mL for C. albicans (Sasidharan and
The results show that *A. niger*, *Aspergillus niger*; *C. a*, *Candida albicans*; *E. c*, *Escherichia coli*; *P. a*, *Pseudomonas aeruginosa*; *S. a*, *Staphylococcus aureus* is effective in killing all the microorganisms tested. Low concentration of *P. cubeba* extract is needed to kill *C. albicans* and *S. aureus* which was 3.2 mg/mL. Low concentration of *P. cubeba* extract is needed to kill *C. albicans* and *S. aureus* which was 3.2 mg/mL followed by *E. coli* (6.4 mg/mL), *P. aeruginosa* (12.8 mg/mL) and *A. niger* (25.6 mg/mL). *P. cubeba* is used as antibacterial, expectorant and gastroprotective agent. The pepper leaf oil which is wasted at present can be utilized against these microorganisms instead of costly synthetic chemicals.

The results show that *P. cubeba* L. extract is more effective in killing all the microorganisms tested. Low concentration of *P. cubeba* extract is needed to kill *C. albicans* and *S. aureus* which was 3.2 mg/mL followed by *E. coli* (6.4 mg/mL), *P. aeruginosa* (12.8 mg/mL) and *A. niger* (25.6 mg/mL). *P. cubeba* is used as antibacterial, expectorant and gastroprotective agent.
The development of resistance in common foodborne pathogens and emergence of new foodborne pathogens intrinsically resistant to the currently available antibiotics demonstrates the urgent importance of identifying novel natural antimicrobial agents. There will be an increasing need for microbial inhibiting substances from plants. The traditional medicinal plants represent a reservoir of antimicrobial agent. Present study shows, *P. cubeba* extract shows the most potent antimicrobial activity against five standard species microorganisms. Therefore, *P. cubeba* extract and its compounds might be potentially valuable as a natural food preservative.

**Conclusion**

The development of resistance in common foodborne pathogens and emergence of new foodborne pathogens intrinsically resistant to the currently available antibiotics demonstrates the urgent importance of identifying novel natural antimicrobial agents. There will be an increasing need for microbial inhibiting substances from plants. The traditional medicinal plants represent a reservoir of antimicrobial agent. Present study shows, *P. cubeba* extract shows the most potent antimicrobial activity against five standard species microorganisms. Therefore, *P. cubeba* extract and its compounds might be potentially valuable as a natural food preservative.

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**Table 2. Minimum inhibitory concentration (MIC) (mg/mL), minimum fungicidal concentration (MFC) (mg/mL), and minimum bactericidal concentration (MBC) (mg/mL) of edible medicinal plant extracts (EMPE) on five standard species for food preservative**

<table>
<thead>
<tr>
<th>Sample and microorganism species*</th>
<th><em>A.n</em></th>
<th><em>C.a</em></th>
<th><em>E.c</em></th>
<th><em>P.a</em></th>
<th><em>S.a</em></th>
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<tbody>
<tr>
<td><em>Alpinia galanga</em> (L.) Blume</td>
<td></td>
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<td>12.8</td>
<td>25.6</td>
<td>25.6</td>
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<td><em>Abreu's precatorius</em> L.</td>
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<td><em>Boesenbergia rotunda</em> (L.) Mansf.</td>
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<td><em>Curcuma longa</em> L.</td>
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<td><em>Curcuma aeruginosa</em> Roxb.</td>
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<tr>
<td><em>Curcuma xanthorrhiza</em> Roxb.</td>
<td>12.8</td>
<td>25.6</td>
<td>3.2</td>
<td>6.4</td>
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<td><em>Curcuma mangga</em></td>
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<td><em>Coriandrum sativum</em> L.</td>
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<td>6.4</td>
<td>12.8</td>
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<td><em>Centella asiatica</em> (L.) Urban</td>
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<td><em>Cinnamomum verum</em> J. Presl.</td>
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<td>25.6</td>
<td>51.2</td>
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<td><em>Cryptocarya masoy</em> Kostern.</td>
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<td></td>
<td>12.8</td>
<td>12.8</td>
<td>51.2</td>
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<tr>
<td><em>Colocasia esculenta</em> (L.) Schott</td>
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<tr>
<td><em>Hippophrora longiflora</em> (L.) G. Don</td>
<td>12.8</td>
<td>51.2</td>
<td>6.4</td>
<td>12.8</td>
<td>25.6</td>
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<td><em>Ligusticum acutilobum</em> S. et Z.</td>
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<td><em>Myristica fragrans</em> Hoult. (nace)</td>
<td>25.6</td>
<td>&gt;51.2</td>
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<td>12.8</td>
<td>51.2</td>
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<td><em>Nigella sativa</em> L.</td>
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<td><em>Orthosiphon aristatus</em> Benth.</td>
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<td><em>Oximium basilicum</em> L.</td>
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<td><em>Piper nigrum</em> L.</td>
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<td>25.6</td>
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<td><em>Psidium guajava</em> L.</td>
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<td><em>Zingiber aromaticum</em> Vahl.</td>
<td>3.2</td>
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<td>6.4</td>
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*A.n, Aspergillus niger; C.a, Candida albicans; E.c, Escherichia coli; P.a, Pseudomonas aeruginosa; S.a, Staphylococcus aureus*
11-1586RU (2012).

References


